

Molecular biomarkers monitoring human skeletal muscle fibres and microvasculature following long-term bed rest with and without countermeasures

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Abstract

The cellular mechanisms of human skeletal muscle adaptation to disuse are largely unknown. The aim of this study was to determine the morphological and biochemical changes of the lower limb soleus and vastus lateralis muscles following 60 days of head-down tilt bed rest in women with and without exercise countermeasure using molecular biomarkers monitoring functional cell compartments. Muscle biopsies were taken before (pre) and after bed rest (post) from a bed rest-only and a bed rest exercise group ($n = 8$, each). NOS1 and NOS3/PECAM, markers of myofibre 'activity' and capillary density, and MuRF1 (E3 ubiquitin-ligase), a marker of proteolysis, were documented by confocal immunofluorescence and immunoblot analyses. Morphometrical parameters (myofibre cross-sectional area, type I/II distribution) were largely preserved in muscles from the exercise group with a robust trend for type II hypertrophy in vastus lateralis. In the bed rest-only group, the relative NOS1 immunostaining intensity was decreased at type I and II myofibre membranes, while the bed rest plus exercise group compensated for this loss particularly in soleus. In the microvascular network, NOS3 expression and the capillary-to-fibre ratio were both increased in the exercise group. Elevated MuRF1 immunosignals found in subgroups of atrophic myofibres probably reflected accelerated proteolysis. Immunoblots revealed overexpression of the MuRF1 protein in the soleus of the bed rest-only group ($> 35\%$ vs. pre). We conclude that exercise countermeasure during bed rest affected both NOS/NO signalling and proteolysis in female skeletal muscle. Maintenance of NO signalling mechanisms and normal protein turnover by exercise countermeasure may be crucial steps to attenuate human skeletal muscle atrophy and to maintain cell function following chronic disuse.

Key words exercise countermeasure; human skeletal muscle; microgravity; nitric oxide synthase; spaceflight; ubiquitin-proteasome pathway.

Introduction

Neuromusculoskeletal system plasticity and the underlying biomolecular and genetic mechanisms of plasticity changes are fundamental aspects of skeletal muscle biology and adaptation (Billeter et al. 1997; Fluck & Hoppeler, 2003). Whole-body immobilization in clinical settings or microgravity-induced unloading during spaceflight result into various detrimental effects upon multiple body systems (body deconditioning) with regressive/atrophic mechanisms primarily observed by altered neuromusculoskeletal system structure and function, e.g. muscle fatigue and weakness

(Hakkinen, 1994). As a result, an overall impaired performance control on Earth or in microgravity in space can be observed (Baldwin et al. 1996).

In addition to investigations on the cellular, molecular and genetic regulators that may account for skeletal muscle adaptation (Booth et al. 2002; Stewart & Rittweger, 2006), optimal forms of countermeasure prescriptions must be established to support physical activity and movement control, and to ensure fitness for, for example, locomotor disorders in clinical patients and rehabilitation (Dietz & Duysens, 2000), or performance control of astronauts in outer space (Adams et al. 2003). The 6° head-down tilt bed rest model is an accepted functional analogue to microgravity environments (Adams et al. 2003). Bed rest provides a scientifically controlled and ethically acceptable experimental set-up for studying atrophy and plasticity as well as maladaptation of the neuromusculoskeletal system in sufficient numbers of healthy volunteers in specialized facilities or university hospitals (Blottner, 2007). As the

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amount of loss in skeletal muscle mass occurs exponentially in relation to the duration of muscle in bed rest (Bloomfield, 1997) and as differences of, for example, skeletal muscle composition and exercise capacity seem to occur between male and female subjects (Yamamoto et al. 1997; Koryak, 1998) the cellular responses of skeletal muscle functional compartments to chronic unloading conditions need to be determined for both genders in controlled bed rest studies of various durations.

In the WISE Study 2005, i.e. female 60 days head-down tilt bed rest, our principal aim therefore was to determine the effects of chronic muscle disuse and exercise countermeasure by high-resolution confocal immunohistochemistry and quantitative immunoblot analyses using specific biomarkers that should monitor altered expression of specific proteins relevant to functional skeletal muscle compartments. Muscle biopsies were taken before and after bed rest from the mixed fast/slow-type vastus lateralis (VL) and the mainly slow-type soleus muscle (SOL). Subject-matched biopsies were analysed for the expression of nitric oxide (NO)-synthase (NOS), an indicator of 'activity' as previously shown in a male bed rest study (Rudnick et al. 2004). The NO-generating enzymes are abundantly found in human skeletal muscle fibres (Nakane et al. 1993) and at the endothelial layer of the muscular microvasculature (Rudnick et al. 2004). Intramuscular NO signalling pathways are associated with muscle activity under normal and chronic exercise conditions (Fujii et al. 1998; Tidball et al. 1998; Vassilakopoulos et al. 2003) as well as in limb blood flow and microperfusion (Maxwell et al. 1998; Lau et al. 2000). Yet, expression of NOS in female skeletal muscle in response to extended bed rest immobilization with and without exercise remains unknown.

Disuse-induced atrophy is characterized by considerable protein degradation (Ferrando et al. 2002) and possibly activation of the ubiquitin (Ub)-proteasome pathway (Jackman & Kandarian, 2004). Our second aim therefore was to monitor proteolysis in atrophic vs. trained muscle biopsies at the level of muscle fibres in bed rest with the proteolysis biomarker protein muscle-specific RING finger type-1 (MuRF1), a sarcomere-associated E3 Ub-ligase that is upregulated by multiple types of muscle atrophy (Glass, 2003). We hypothesized that in the bed rest-only group elevated MuRF1 levels found in biopsies and in individual myofibres might reflect accelerated proteolysis in skeletal muscle. Exercise performed during bed rest might, in turn, result in normal MuRF1 levels, suggesting maintenance of relatively normal protein turnover rates thus indicating less atrophy in disused human muscles. Specific biomarkers may thus help to understand further some of the cellular responses to chronic muscle inactivity and may be used to evaluate the efficacy of exercise countermeasure protocols at the level of muscle fibres in conditions of clinical bed rest or human spaceflight.

Material and methods

Ethical considerations

This study was based on two needle biopsies from each subject [i.e. one before (designated preBR) and one at the end of the bed rest period (designated postBR)] of the VL and SOL muscle from the right leg of a bed rest-only (BR-CTRL) and a bed rest exercise (BR-EX) group. Each volunteer gave informed written consent to the muscle biopsies. Biopsy procedures were approved by the local Ethical Committee [le comité consultative de protection des personnes dans la recherche biomédicale de Toulouse I (CCPPRB), France]. The study rules of WISE were in accordance with the Declaration of Helsinki, 1964, and the Good Clinical Practice Recommendations of the International Conference on Harmonisation.

The bed rest campaign

The WISE 60-day strict anti-orthostatic 6° head-down tilt (HDT) bed rest campaign took place in 2005 at the Clinique Spatiale MEDES (www.medes.fr, CHU Rangueil University Hospital, Toulouse, France). The study was part of an international collaboration between space agencies, i.e. the ESA (www.spaceflight.esa.int), NASA (www.nasa.gov), CSA (www.space.gc.ca) and National Space Agency of France, CNES (www.cnes.fr). Twenty-four healthy age- and weight-matched non-smoking women (Table 1) were recruited from nine different European countries and randomly assigned to three different groups (each $n = 8$), i.e. a bed rest-only group (BR-CTRL) without training, a nutrition group (NUTR) subjected to a special amino acid diet ($n = 8$, not included in the present study) and a training group (BR-EX) performing exercise during bed rest (i.e. fly-wheel plus LBNP treadmill, see below). All groups underwent strict minus 6° head-down tilt bed rest in two subsequent campaigns in 2005. All candidates gave informed consent to participate in this study. At the time of being recruited, the volunteers were neither involved in regular intense or systematic training programmes nor under hormonal contraception medication for at least 3 months prior to or during the WISE study. All subjects were kept in strict anti-orthostatic supine position except for during meals

Table 1 Pre bed rest anthropometric data

	Control	Exercise
Age (years)	34 ± 4	33 ± 4
Height (cm)	162.3 ± 6.2	164 ± 7.1
Weight (kg)*	56.3 ± 3.0	58.4 ± 6.4
BMI (kg m ⁻²)	21.20 ± 0.93	21.75 ± 1.52

*Obtained during study familiarization; mean ± SD.

(elevation on one elbow allowed). Transportation, showering, toileting as well as training procedures were restricted to the bed rest recumbent position. To ensure compliance, video surveillance and pressure-sensitive mattresses monitored the volunteers at all times with the only exception being for daily bathroom and shower activities. Except for training interventions of the exercise group, no physical activity was allowed during bed rest. Physiotherapy, massage and ankle circumduction movements as well as medical checks were provided daily. All subjects received identical nutrition calculated on the basis of pre-study body weights according to the WHO standards in order to avoid significant weight variations due to over/undernourishment.

Bed rest groups

The bed rest-only group (BR-CTRL) consisted of eight healthy female subjects (Table 1) adherent to a strict minus 6° head-down tilt BR position without any further exercise. The bed rest position results in the mechanical unloading preferentially of lower limb muscle groups (e.g. thigh and calf) as well as in a cranial blood volume/fluid shift from lower to upper body with cardiovascular deconditioning, which ultimately leads to the exponential development of disuse-induced skeletal muscle and cardiac atrophy during ongoing bed rest (LeBlanc et al. 1997).

The bed rest exercise group (BR-EX) consisted of eight healthy female volunteers (Table 1) who in addition to bed rest were engaged in a combined resistive and aerobic muscle training protocol during the 60-day study. Resistive exercise in this group was performed using an inertial flywheel ergometer (FEW; YoYo® Technology Inc., Sweden) for use in bed rest or spaceflight (Berg & Tesch, 1998). The exercise group thus trained the knee extensors of the thigh and plantar flexors of the calf muscle groups using supine squat and calf press exercises at maximal strength as previously described (Alkner & Tesch, 2004). A total of 19 FWE sessions were scheduled for each subject approximately every third day starting on day 2 of bed rest. Ten minutes of light supine cycling and submaximal supine squat and calf press repetitions were completed as warm-up. The supine squat exercise consisted of four sets of seven maximal concentric and eccentric repetitions, while the calf press exercise consisted of four sets of 14 maximal concentric and eccentric repetitions with 2 min resting time between each sessions. Force and flywheel rotational velocity were measured, and work and power were calculated throughout each repetition as in a previous 90-day study conducted in males (Alkner & Tesch, 2004).

Aerobic exercise was performed in the BR-EX group during the 60-day bed rest period three times a week (between resistive FWE intervals) using a supine lower body negative pressure (LBNP) treadmill device. In principle, the device consists of a vacuum chamber that provides an attractive

force between subject and a vertical treadmill to substitute for the effects of gravity (e.g. axial load of about 60–65% of their body weight) in upright exercise (Watenpaugh et al. 2000). The negative pressure required to produce 1.0× body weight (BW) and to normalize heart rate to upright exercise in 1g was approximately 48–55 mmHg. For use in supine bed rest position, a sling supported the subject's upper body with a solid back support outside the chamber, and the hips were supported with a soft sling inside the chamber. Soft Velcro-reinforced fabric cuffs were placed above the knees and ankles. A bungee cord connected the cuffs at their respective positions through pulleys suspended from the inside top of the chamber. In this way, the legs counterbalanced each other and minimized the work required to move against gravity. The distance between the treadmill and the chamber opening was adjusted such that the iliac crest was positioned at the level of the opening when the subject's feet were pressed lightly against the treadmill with legs fully extended. Three days per week, subjects performed 40 min of dynamic treadmill exercise, followed by 10 min of resting LBNP. This protocol was similar to that which successfully preserved upright exercise capacity during 30 days of bed rest (Lee et al. 1997). Target speeds to achieve various exercise intensities (i.e. 40–80% of preBR VO_2 peak) were prescribed based upon a linear relationship between treadmill speed and VO_2 determined during the preBR graded exercise test performed in upright posture. LBNP produced foot-ward force of 1.0× BW at the start of BR and was increased up to 1.2× BW during BR, based upon subject tolerance to the exercise countermeasure. During the course of the 60-day BR, 29 exercise sessions were prescribed for each subject. Of the exercise sessions performed, the mean exercise time was 50 ± 2 min on each training day. Across all exercise sessions completed, the average LBNP was 52 ± 3 mm Hg, which corresponded to a mean loading of 1.0 ± 0.1 BW sufficient to produce an adequate weight-bearing muscle load and cardiovascular support for supine treadmill exercise. A detailed description of the LBNP device and exercise protocol during the WISE Study has been published elsewhere (Dorfman et al. 2007).

Muscle biopsy

One day before start of strict BR (BDC-1, designated as preBR), and at day 59 of BR (BR59, designated as postBR), two biopsies were taken from each volunteer, one from mixed/fast-type VL, the lateral aspect of the hip muscle (m. quadriceps femoris), and one from the mainly slow-type SOL muscle, the deep aspect of the calf muscle (triceps surae) of the right leg while still in the bed with the percutaneous needle biopsy technique (Bergstrom, 1979) combined with suction (Evans et al. 1982). For immunohistology, small samples of approximately 30 mg per biopsy were orientated

with their myofibres running longitudinally under binocular control in 5-mm-diameter silicone tubes filled with embedding medium (OCT; www.mediate.ch). The samples in tubes were immediately frozen in isopentane precooled with liquid nitrogen and stored at -80°C . For biochemistry, samples of ~ 30 mg per biopsy were immediately shock-frozen in liquid nitrogen and stored at -80°C . The biopsy protocol for WISE was essentially the same as for previous bed rest studies (LTBR 2001, BBR 2003) including a subject-matched analysis (i.e. the pre vs. post biopsies were from identical volunteers) in order to balance the anticipated intersubject variability (Rudnick et al. 2004).

Immunohistochemistry and confocal laser scanning (CLS) microscopy

Frozen samples were removed from the silicone tubes and serial cross-sections were cut at $10\text{ }\mu\text{m}$ of thickness in a cryostat (LEICA CM 3000; www.leica-microsystems.com), mounted to protein-coated and coded slides and subjected to various immunohistochemical staining protocols. We used primary monoclonal antibodies against the neuronal NOS type-1 (NOS1) isoform (n-terminal amino acids 2–300; www.scbt.com), human endothelial NOS3 (c-terminal amino acids 1025–1203) isoform (bdbiosciences.com), monoclonal anti-PECAM-1 (anti-human platelet endothelial cell adhesion molecule, anti-human CD31, clone # JC70A, diluted 1 : 25; www.dako.com). Primary antibodies were diluted 1 : 1000–1 : 1500 and incubated overnight at 4°C followed by incubation with Alexa 488/Alexa 555-conjugated goat anti-mouse secondary antibody diluted 1 : 200–1 : 4000 for 1 h at room temperature (RT; www.invitrogen.com). We also used a polyclonal goat-anti-muscle RING zinc finger protein-1 (MuRF1) found in striated muscle (www.abcam.com) diluted 1 : 100 overnight at 4°C , followed by donkey anti-goat ALEXA 555 (1 : 1500) for 1 h at RT. Controls include incubation of cryosections with secondary antibody only and antibody blocking peptide if available. In order to test for cross-reactivity of secondary antibodies, various secondary detection antibodies from different hosts were checked and finally chosen for double immunohistochemistry. Labelled cryosections were inspected by routine light and epifluorescence microscopy (www.zeiss.com/micro) and by a high-signal resolution LEICA SP-2 CLS microscope equipped with a multilaser system (Ar laser, 458–514 nm; HeNe laser, 543 nm; HeNe laser, 633 nm) under standardized laser scanning presettings [i.e. 1024×1024 scan format, 150–350 nm² voxel size, Ar/HeNe laser intensities (25% and 50% intensity) and gain settings (500 ± 10.0 V)] of the three photomultipliers (www.leica-microsystems.com/ Confocal_Microscopes). Multi-channel detection was made by the sequential scan mode to avoid overlapping fluorescence emission signal detection in double-immunostained sections, as previously described (Rudnick et al. 2004).

Myofibre type composition, cross-sectional area and NOS1 intensity

The fast and slow fibre type composition of muscles were analysed by double labelling of cryosections with monoclonal anti-skeletal fast-type myosin (clone My32, diluted 1 : 1500), a selective type II marker cross-reactive with antigens IIA, IIB and IIC/X, and slow-type myosin heavy chain (clone NOQ7.5.4D, 1 : 1000; www.sigmaaldrich.com), a selective type I marker. Both antigens are preferentially expressed in fast-twitch type II and slow-twitch type I myofibres. The primary antibodies were detected by secondary goat anti-mouse Alexa 555 (1 : 2000) and/or Alexa 488 (1 : 4000) for 1 h at RT, respectively (www.invitrogen.com). Only major populations of myofibres I and II with clear borders were screened. Therefore, transient fibre types and subclassifications based on myofibrillar pH stability/lability of ATPase and MHC content (Staron, 1997) were not determined in the present study. Similar primary and secondary antibody dilutions were used for double immunostaining protocols identifying sarcolemma NOS1 immunoreactivity at individual myofibre types I vs. II. The mean cross-sectional areas (CSAs) of myofibres I and II were measured in digitized images of NOS1/fast MyHC or slow MyHC single and double-stained cryosections by use of the LEICA image analysis software (release #2.61.1537). A few myofibres expressing only faint My-32 immunolabelling (hybrid fibres) were excluded from all measurements. Myofibre-specific CSA values (μm^2) were given as percentage relative difference (% change) of pre bed rest (arbitrarily set as zero baseline %) vs. post bed rest data ($\Delta\%$ from zero baseline) using the subject-matched analysis. The relative fluorescence intensity of NOS1-immunolabelled sarcolemma of myofibres I and II was determined by pixel-intensity measurements in digitized image scans as previously described (Rudnick et al. 2004). Briefly, a small rectangular box (region of interest, ROI) of approximately 2400 pixels ($40 \times 8\text{ }\mu\text{m}$ or $320\text{ }\mu\text{m}^2$ area) was created and positioned via the cursor over four different sarcolemma portions on the basis of individual cross-sectioned myofibre I or II profiles in digitized images. Each ROI included only sarcolemma portions of the same myofibre type in order to get separate measures from the two different myofibre types I and II, and to avoid bias in the placement of the ROI (i.e. covering two adjacent membrane portions of neighbouring type I and II profiles). Pixel intensities were then calculated from each ROI given as mean arbitrary units (a.u.) \pm SEM.

The microvascular network of skeletal muscle

Immunofluorescence detected endothelial-derived NOS3 in the microvasculature and quantitative detection of the capillarity was done by NOS3/PECAM-1 (CD31) co-immunostaining followed by quantitative determination of the capillary-to-fibre ratio (C/F ratio) as a global representation

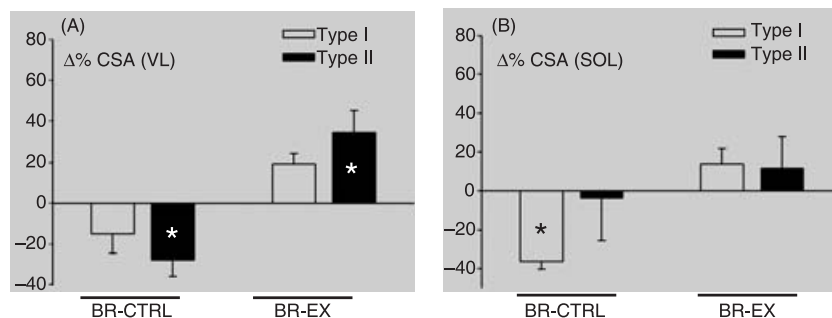


Fig. 1 Quantitative determination of the cross-sectional area (CSA) of vastus lateralis (VL) and soleus (SOL) slow-type (I) and fast-type (II) muscle fibres of women in 60-day bed rest without (BR-CTRL) and with exercise (BR-EX) countermeasure. BR-CTRL group (bed rest-only): the relative size of myofibres is reduced in (A) VL ($n = 7$) and (B) SOL ($n = 5$) as compared with baseline (subject-matched pre bed rest values set as 0%). BR-EX group (bed rest plus resistive and aerobic exercise): the CSA values are increased in (A) VL ($n = 8$) and maintained in (B) SOL ($n = 5$) as compared with baseline (subject-matched pre bed rest values set as 0%). Significance at $P < 0.05$ (*).

of the capillary supply in arbitrary groups of cross-sectioned myofibres. From each sample, three immunostained cryosections were digitally scanned by CLS under standardized conditions ($\times 20$ objective magnification, 150×150 nm voxel size, 1024×1024 scan format). In three separate cryosections, NOS3/PECAM-1 co-immunostained capillary profiles located at endomysial connective tissue were counted manually by two independent investigators in selected artefact-free ROIs (area of about $97\,500 \mu\text{m}^2$) on an individual-fibre basis in randomly distributed blocks of 15 cross-sectioned fibres. The total capillary number per area was subdivided by the total fibre number (triplicate determination) per area and expressed as the mean C/F ratio for each muscle and subject per group.

Western blot analysis

Lysates of subject-matched skeletal muscle biopsies from bedridden women were separated by electrophoresis (Protean System, www.bio-rad.com) as previously described (Rudnick et al. 2004). Briefly, each gel was loaded with preBR and subject-matched postBR protein samples (7.5 or 15 μg) from candidates of the BR-CTRL and BR-EX group (final concentration 1 mg mL^{-1}). Remaining lanes were loaded with prestained broad-range standard molecular weight markers (www.bio-rad.com) and positive controls for NOS1 protein (160 kDa, 7.5 μg load) from mouse brain (www.bdbiosciences.com), or with 5 μg of human endothelial cell lysates (140 kDa, 1 mg mL^{-1} , BD). Transblotted membranes were blocked overnight at 4°C with blocking buffer (4 % non-fat milk powder) in TBST (20 mM Tris base, 500 mM NaCl, 0.05% Tween 20, pH 7.4). Protein bands were immunolocalized on transblots with primary monoclonal anti-NOS1 (www.scbt.com, 1 : 750) in blocking buffer for 1 h at RT followed by alkaline phosphatase (AP)-conjugated secondary rabbit anti-mouse antibody incubation (www.dakogmbh.de, diluted 1 : 500) for 1 h at RT, and polyclonal goat anti-muscle-specific ring finger protein-1 (i.e. sarcomere-associated protein) MuRF1 (peptide near C-terminal, sc-27642, SCBT, 1 : 150) followed by secondary

AP-conjugated chicken anti-goat (SCBT, 1 : 500). Immunostained membranes were developed by use of an NBT/BCIP/1 mM levamisole colour detection kit (www.piercenet.com). Quantifications of NOS1 or MuRF1 antigens were made for each sample in triplicate by determination of the relative optical density (OD) of immunostained protein bands by densitometry scanning (GS-800, Quantity-One™ software, www.bio-rad.com). Mean OD values were expressed as relative percentage difference of postBR samples vs. subject-matched preBR samples (mean OD values arbitrarily set as 0%) as plotted in the graphs.

Statistics

All data were expressed as mean \pm standard error of the mean (SEM). Possible differences among groups were evaluated by ANOVA. Comparisons between two means were based on paired Student's *t*-test after variance analysis. Significance for all tests was determined at the $P < 0.05$ level, or as elsewhere given in the figures.

Results

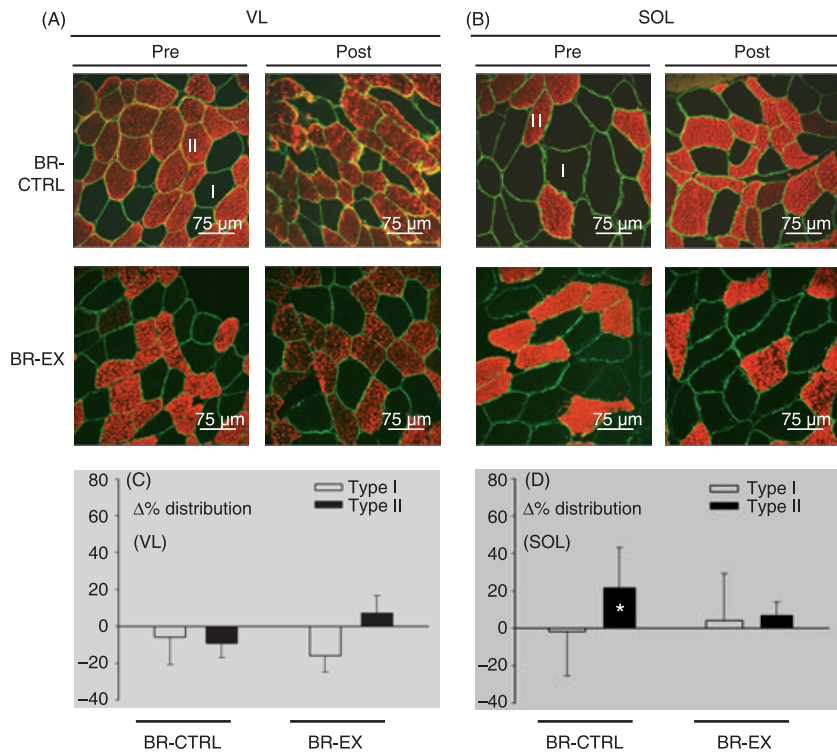
Exercise in bed rest mainly preserved size of fast myofibres II in VL and SOL

For size determination of the muscle fibres we performed quantitative morphometrical measurements on cross-sectioned profiles (CSA) of myofibres I and II identified by slow/fast MyHC/NOS1 co-immunostaining (Fig. 1A,B).

BR-CTRL

In VL, reduced myofibre size profiles were observed after bed rest (Fig. 1A), which is reflected by significantly decreased post CSA values in both myofibres I and II (-20% and -30% respectively, compared with subject-matched pre CSA values set as zero baseline). In SOL, overall reduced myofibre size profiles were also documented after bed rest (Fig. 1B). Here, significantly altered post CSA values were documented

Fig. 2 Distribution patterns of myofibre type I/II in vastus lateralis (VL) and soleus (SOL) of women in bed rest without (BR-CTRL) and with (BR-EX) exercise. (A,B) Detailed areas of representative image pairs of VL and SOL with myofibre type II (fMyHC, red) and sarcolemma co-immunostaining (NOS1, green). BR-CTRL group: in VL ($n = 7$), only minor changes were seen in the distribution of myofibres I and II. BR-EX group: the VL ($n = 8$) showed altered myofibre type patterns (more myofibres II than I). BR-CTRL group: in detailed areas of representative image pairs of SOL ($n = 5$), but not VL ($n = 7$), the myofibre type pattern was significantly altered after bed rest (relatively more myofibres II than I). BR-EX group: in SOL ($n = 5$) and VL ($n = 8$) the relative myofibre type pattern after bed rest was similar to normal (pre values). (C,D) Statistical analysis of the relative change in VL (C) and SOL (D) myofibre type distribution. Significant changes were observed only in SOL type II myofibres of the BR-CTRL group. The subject-matched pre bed rest values were set as 0% baseline. Significance at $P < 0.001$ (*), paired Student's t -test after variance analysis.



only in myofibres I (-35% vs. pre), with minor changes of CSA values in myofibres II (-5% vs. pre) as compared with pre CSA values (Fig. 1B).

BR-EX

In VL, the fibre profile size was changed (Fig. 1A) as reflected by the increased CSA values in both myofibre types I and II (by 20% and 30%) as compared with subject-matched pre values. In SOL, the size of the myofibre types I and II profiles was largely maintained with a trend to increased profile size, i.e. hypertrophy, in the range 10–15% vs. baseline (Fig. 1B,D).

Exercise preserved relative distribution patterns of myofibres I vs. II

As the myofibre type distribution patterns may be altered by muscle disuse or activity, we determined the changes in the relative distribution of slow and fast-type myofibres ($\Delta\%$ distribution) in cross-sectioned biopsies in both BR-CTRL vs. BR-EX groups before and after bed rest (Fig. 2).

BR-CTRL

In VL, small changes of the myofibre pattern I vs. II (c. 5–10% change) were documented after bed rest as compared with 0% baseline representing subject-matched pre levels (Fig. 2). In SOL, a significant shift in the distribution patterns of myofibres I and II (i.e., slow > fast) was clearly documented by confocal immunofluorescence and confirmed by quantitative counts of fMyHC-immunoreactive

profiles (red fluorescence) vs. non-labelled profiles denoted as slow myofibres I (Fig. 2).

BR-EX

In VL, by contrast, relatively more myofibres II than I were detectable at the end of bed rest (Fig. 2). In SOL, however, a similar myofibre type shift was not detectable at the end of bed rest (Fig. 2). Quantified data are presented as bar graphs (Fig. 2B) representing percentage change ($\Delta\%$) of myofibres I and II vs. baseline (subject-matched pre values).

Exercise increased NOS1 expression in both slow and fast myofibres

The altered myofibre membrane expression of the NOS1 biomarker can be readily seen in confocal images of pre vs. post biopsies by variable membrane signal intensities (immunofluorescence) in cross-sectioned myofibre profiles (Fig. 3A). Thus, we measured such changes by quantitative pixel analysis of NOS immunofluorescence signals at defined subsets of membrane portions as outlined by rectangular ROIs (2400 pixels within $320 \mu\text{m}^2$) using image scans of cryosections from the biopsies. Only membranes in well-defined subgroups of either myofibres I or II profiles were measured in order to avoid detection of overlapping membrane signals from neighbouring myofibres (Fig. 3A). Quantitative data of VL or SOL are shown as bar graphs (Fig. 3B). In the BR-CTRL group, reduced sarcolemma NOS1 intensity levels were found in myofibres of VL. In SOL myofibres the NOS1 intensity was significantly decreased

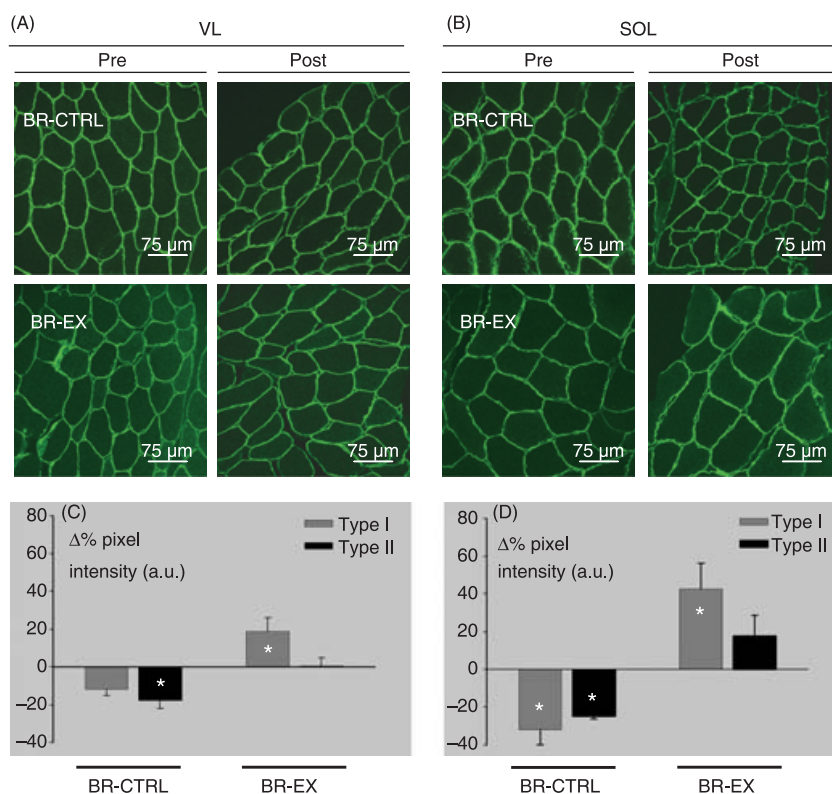


Fig. 3 Nitric oxide synthase type-1 (NOS1) immunoreactivity and fluorescence intensity measurements at myofibre plasma membranes of biopsies without (BR-CTRL) and with (BR-EX) exercise before and after 60 days of bed rest in women. (A,B) Representative subject-matched pairs of confocal laser scan microscopy images of NOS1-immunostained cryosections before (pre) and after (post) bed rest. BR-CTRL group: decreased NOS1 immunoreactivity is seen in detailed images of post vs. pre bed rest VL and SOL. BR-EX group: increased NOS1 immunoreactivity is seen in post vs. pre bed rest VL and SOL. (C,D) Quantitative pixel-image analysis (arbitrary units = a.u.) of post vs. pre bed rest sarcolemma NOS1 immunoreactivity (pre set as 0% baseline). BR-CTRL group: in both VL (C) and SOL (D), reduced membrane NOS1 immunofluorescence signals (pixel intensity post vs. pre bed rest) were present in slow (type I) and fast (type II) fibres. BR-EX group: in VL (C) and SOL (D), increased membrane NOS1 immunofluorescence signals (pixel intensity post vs. pre bed rest) were found, but only in myofibres I of VL and SOL, whereas NOS membrane signals of myofibres II were not changed. For sample numbers see Fig. 2. (*) Significance at $P < 0.001$, paired Student's *t*-test after variance analysis.

as compared with subject-matched pre baseline levels (Fig. 3). In the BR-EX group, the sarcolemma NOS1 intensity levels were clearly maintained (VL) or even overexpressed (SOL) after bed rest as compared with the pre baseline levels (Fig. 3).

Changes of MuRF1 proteolysis biomarker in VL and SOL

In both VL and SOL, MuRF1 immunoreactivity was detected by confocal analysis in the cytosolic/myofibrillar compartment of myofibres (Fig. 4). Apart from a low to moderate baseline expression in muscle fibres, strong cytosolic immunofluorescence MuRF1 signals were detected in subpopulations of atrophic myofibres in both VL and SOL after bed rest (post), which were not seen in subject-matched pre samples (Fig. 4A, insets). However, no clear correlations between MuRF1 expression and myofibre types were found using MuRF1/MyHC co-immunolabelling techniques in either BR-CTRL or BR-EX groups (data not shown).

Immunoblot analysis of subject-matched biopsy lysates revealed a major MuRF1-immunoreactive band (arrow) in both the BR-CTRL and the BR-EX groups (Fig. 4B). From each pair (i.e. subject-matched pre vs. post), the MuRF1-immunoreactive band was analysed by densitometry (Fig. 4C). After bed rest, significant changes of MuRF1 protein levels were not found in VL of the BR-CTRL or the BR-EX group as compared with pre levels. In untrained SOL

(BR-CTRL), significant changes of MuRF1 protein levels were observed (> 35% vs. baseline). However, in trained SOL of the BR-EX group, MuRF1 levels were largely maintained following bed rest as compared with pre levels (Fig. 4C).

Changes in the microvasculature patterns and C/F ratio

In subject-matched pre vs. post biopsies of VL and SOL, immunostaining was performed using an endothelial-specific NOS3 isoform antibody that was combined with the endothelial marker PECAM-1 to identify the microvascular profile pattern, including the capillary structures at the endomysial interface between skeletal muscle fibres (Fig. 5). As expected, a nearly 1 : 1 overlapping immunostaining pattern was observed with both biomarkers (Fig. 5, insets, lower right panel). Virtually no changes in either NOS3 intensity or capillary density patterns of the female hip and calf skeletal muscle of the BR-CTRL group were seen as compared with subject-matched pre values (see lower panel insets of Fig. 5). In the BR-EX group, post bed rest samples revealed an altered capillary density pattern as compared with pre bed rest samples from this group (Fig. 5). Histomorphometric counts of immunostained capillary structures within predefined groups of myofibres were calculated and expressed as the C/F ratio for each muscle and subject group (Table 1). In the BR-EX group, the C/F ratio was significantly increased as compared with the BR-CTRL group, suggesting the presence of a globally

Fig. 4 MuRF1 immunofluorescence and immunoblot expression in VL and SOL without (BR-CTRL) and with (BR-EX) exercise before and after bed rest in women. (A) Overall distribution of confocal MuRF1 immunofluorescence in VL and SOL after 59 days of bed rest (BR-CTRL). Insets show robust immunofluorescence of individual myofibres found in both VL and SOL, thus monitoring accelerated protein turnover/ degradation at the myofibre level. (B) Western blot analysis of paired subject-matched biopsy lysates showing MuRF1-immunoreactive bands (arrow) in parallel lanes (pre vs. post bed rest). An increase in the MuRF1-immunoreactive band was detectable only in SOL after bed rest (BR-CTRL). (C) Densitometric analysis of MuRF1-immunoreactive protein bands ($\Delta\%$ change post vs. pre bed rest). BR-CTRL group: significant increased MuRF1 levels were found only in SOL, but not in VL, after bed rest. BR-EX group: post bed rest MuRF1 levels did not differ significantly from pre bed rest values. (*) Significant at $P < 0.05$.

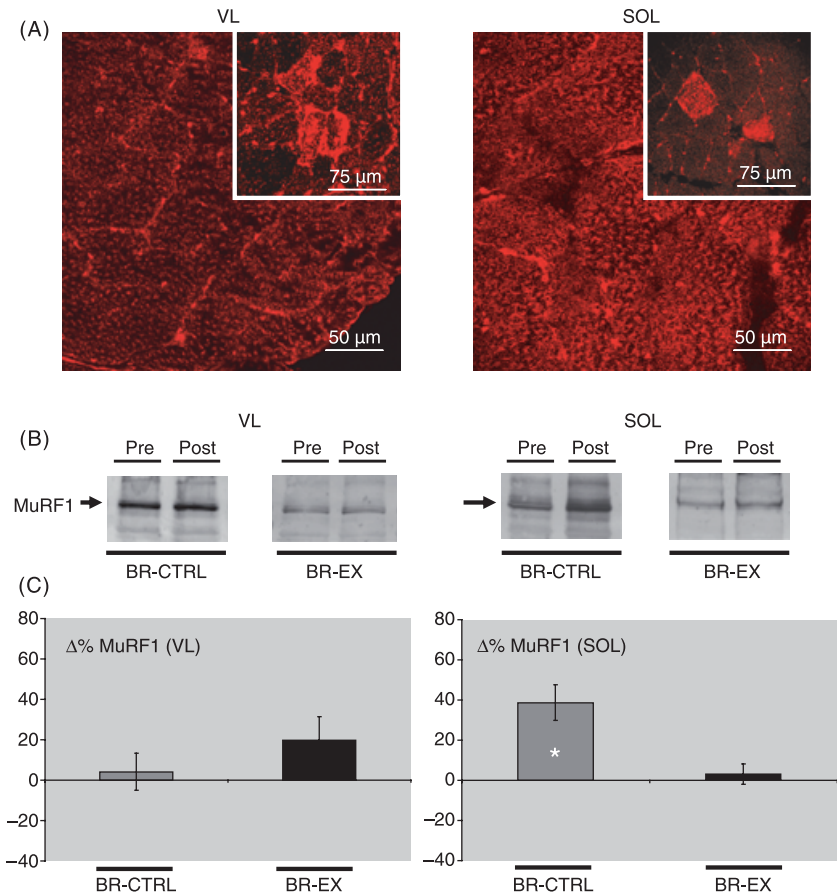


Fig. 5 Capillary density visualized by NOS3/ PECAM immunomarkers in subject-matched pre vs. post bed rest biopsies of the BR-EX group. The immunostaining pattern of NOS3 (green fluorescence) of the microvasculature network and capillaries was different in both VL (upper panel) and SOL (lower panel) as compared with the subject-matched pre bed rest patterns (cf. C/F ratios in Table 2). Insets show higher magnification confocal images of myofibre perpendicular profiles surrounded by NOS3/PECAM co-immunostained capillaries (NOS3, red), and a merged red/green image (NOS3/PECAM, yellow) used for quantification of C/F ratio (Table 2). In the BR-CTRL group, the capillary distribution was unchanged (data not shown).

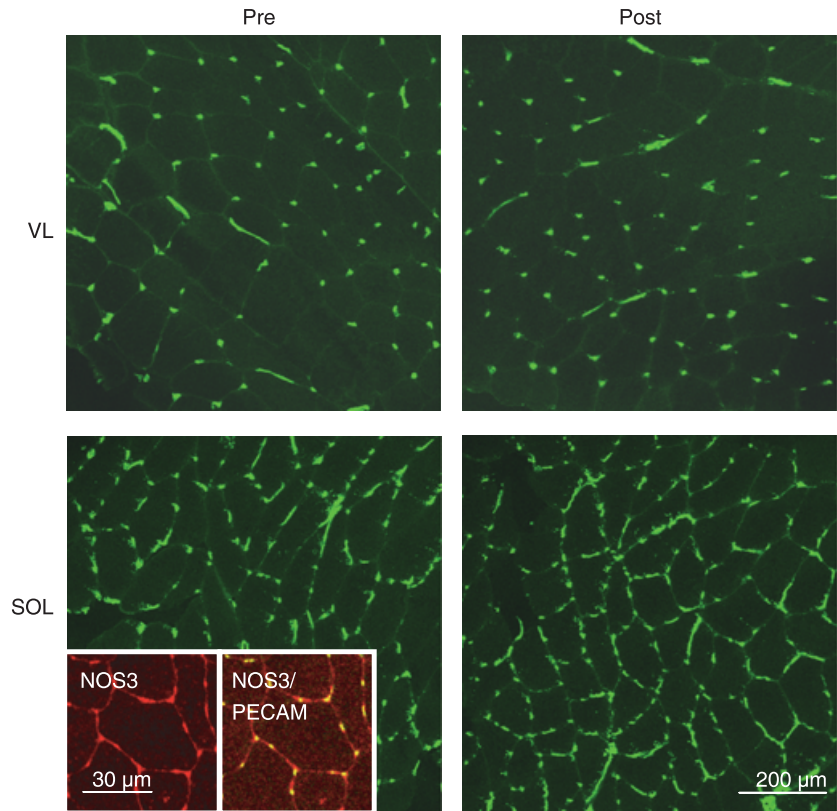


Table 2 Capillary-to-fibre ratios of trained and non-trained VL and SOL muscle in female bed rest (BR)

Muscle/BR group	preBR	postBR	<i>P</i> value
VL/BR-CTRL	1.55 ± 0.21	1.58 ± 0.20	NS
VL/BR-EX	1.69 ± 0.17	2.07 ± 0.38	0.024*
SOL/BR-CTRL	1.96 ± 0.53	1.61 ± 0.19	NS
SOL/BR-EX	1.81 ± 0.17	2.25 ± 0.40	0.05*

Values shown are mean ± SD.

*Significant changes (postBR vs. preBR) compared with subject-matched subjects ($P < 0.05$).

NS, not significant; VL, vastus lateralis; SOL, soleus muscle.

altered capillary network, probably supporting the micro-perfusion capacity for both VL and SOL using the resistive plus aerobic countermeasure protocol during bed rest.

Discussion

The principal new findings of this study were as follows: (1) prolonged bed rest without exercise resulted in atrophy of skeletal myofibres in lower limb muscles and a slow-to-fast shift of the soleus muscle in women; (2) the myofibre size, the slow/fast phenotype composition as well as the muscular NO-signalling pathway of VL and SOL muscle were maintained following a combined resistive and aerobic exercise protocol during bed rest; (3) MuRF1 is overexpressed in the SOL of bed rest subjects only and maintained to almost normal levels in trained VL and SOL after bed rest; and (4) NOS and MuRF1 are useful in monitoring morphological and biochemical changes in functional compartments of atrophied vs. trained human skeletal muscle following chronic disuse in bed rest.

Structural and biochemical responses of female skeletal muscle in bed rest

Changes in the morphology and phenotype of muscle fibres and the cellular and biochemical pathways in humans can only be evaluated by biopsy samples of skeletal muscle. However, being aware of the variability of muscle fibre areas (Lexell & Taylor, 1989), the possible heterogeneity of the fibre size or even the reproducibility of the biopsy sampling (Mahon et al. 1984) must be considered. Confocal and biochemical analysis of VL and SOL muscle biopsies from the WISE study was always done by subject-matched analysis using perpendicularly cut cryosections and proper tracing of cell borders by immunodetection of membrane-bound molecular complex antigens to minimize any bias in terms of intersubject variability (Rudnick et al. 2004).

Large variations in the fibre type distribution can be found within muscle and between individuals (Staron et al. 1990; Staron, 1997) in women. The normal muscle fibre composi-

tion and performance capacity of women has previously been reported (Campbell et al. 1979). We found that preservation of the myofibre size varied between VL (more type II) and SOL myofibre types (more type I) in trained women in bed rest with a clear fibre type II hypertrophy of VL that was missing in the soleus. A greater plasticity of myofibres II seems to exist that appears to be more pronounced during strenuous training and may result in faster atrophy during detraining (Staron et al. 1991) while myofibres I may also hypertrophy following longer exercise protocols (> 10 weeks).

Rapid muscular adaptations were found in both 6-week trained and non-previously trained women (Staron et al. 1990). The number of hybrid fibres expressing mixed MHC isoforms is decreased following heavy resistive exercise, suggesting a shift of hybrid or IIx toward IIa fibres (Staron et al. 1990). In the present study, myofibre subtypes were not properly detected in terms of IIa, IIb or IIx subclassifications. Nevertheless, at the end of the WISE bed rest period only very few fibres (< 3–5% with both slow and fast MyHC immunoreactivity) were seen. Therefore, the significant changes observed in myofibre phenotype distribution, particularly in trained vs. non-trained SOL of women after prolonged bed rest, may not be due to the presence of large proportions of hybrid fibres. Rather, our results suggest an overall increase in the expression of type I myosin.

The muscular NOS1/NO system. A marker of skeletal muscle activity?

Exercise performed during female bed rest increased sarcolemma NOS1 expression, consistent with the results obtained from the previous male bed rest study (Rudnick et al. 2004). We thus conclude that confocal immunodetection of NOS1 at the myofibre level can be considered as an immunocytochemical marker monitoring skeletal muscle activity or inactivity following extended bed rest in both women and men.

Changes in muscular NOS expression have been previously described in normal and diseased human skeletal muscle. Sarcolemmal NOS1 immunoreaction is absent from dystrophic human muscle (Chao et al. 1996) and highly reduced in atrophic myofibres of various human muscle diseases (Schoer et al. 1997). Increased levels of NOS1 proteins have been reported for skeletal muscle following acute physical exercise protocols in humans (Tatchum-Talom et al. 2000). Molecular assembly or disassembly remodelling mechanisms of the sarcolemmal membrane architecture by muscle activity via mechanotransducing complexes, i.e. focal adhesion kinase (FAK) and paxillin, have been shown previously in animal muscle (Fluck et al. 1999). Similar sarcolemma-associated mechanisms would explain altered NOS1 protein levels as reflected by increased sarcolemmal immunofluorescence found in trained vs. non-trained skeletal muscle of both men and women in bed rest. Regular resistance

exercise performed during prolonged bed rest thus maintained sarcolemmal NOS1 and even upregulated normal, i.e. constitutive, NOS1 protein levels in VL and SOL muscle, suggesting beneficial as well as gender-independent effects of muscle activity on the muscular NOS/NO system. More subtle cellular changes of the NOS/NO system and the effects of regular exercise training imposed on human lower limb skeletal muscles during initial time sequences of extended body immobilization remain to be established in future short- and medium-term (e.g. 5- or 20-day) bed rest studies.

Apart from the known functions of NO in skeletal muscle physiology and pathophysiology, unique biological roles of NO signalling in muscle atrophy and plasticity of gravitation-sensitive muscle species have now been well documented by controlled experimental bed rest and exercise countermeasures in both women (this study) and men (Rudnick et al. 2004). The precise molecular mechanisms whereby NO can modulate the function of, for example, redox-sensitive proteins or pathways by, for example, S-nitrosylation still need to be established for skeletal muscle. Prolonged muscle deconditioning may affect NO signalling, which, in turn, may add directly or indirectly to atrophy mechanisms including functional depression of contractility and force production (Kingwell, 2000). The molecular and functional implications of altered NOS1 expression in muscle cell compartments of trained and non-trained skeletal muscle, however, may be widespread and need further investigation.

Monitoring skeletal muscle atrophy by MuRF1

The presence of elevated MuRF1 levels might reflect high protein turnover/degradation by ATP-driven proteolysis preferentially in disused SOL muscle in women that seemed to be suppressed (i.e. returned to pre bed rest baseline levels) by adequate muscle activity during bed rest. A number of atrophy genes, termed atrogens (MAFbx, MuRF1), are differentially expressed in multiple types of atrophy that may comprise a common atrophy programme (Lecker, 2003). Using a morphological approach to monitor bed rest-induced muscle atrophy, we determined the relative protein breakdown/turnover rates by MuRF1 (anti-E3 Ub-ligase) expression and immunostaining in non-trained vs. trained skeletal muscle. Responses of the Ub-proteasome pathway to changes in muscle activity probably reflect muscular remodelling processes at the level of a non-lysosomal ATP-driven proteolysis that is constitutively active in myofibres under adaptation conditions, but also regulates protein turnover continuously in normally active muscle. A set of E1–3 ligases attach Ub polymers to damaged intracellular proteins, which are targeted to the 26S proteasomes for subsequent degradation (Reid, 2005). MuRF1 (E3 Ub-ligase) belongs to a list of molecular signalling pathway markers that are increased with diminished muscle use and that are upregulated following muscle

denervation, or in spaceflown rats (Bodine et al. 2001; Nikawa et al. 2004).

We found that MuRF1 levels at the end of bed rest were highly upregulated in non-trained SOL (BR-CTRL) and were at normal levels following resistive plus aerobic training of women during bed rest (BR-EX). Similar observations were not made in the trained (BR-EX) vs. non-trained VL (BR-CTRL) women. We therefore conclude that altered protein turnover rates detected by MuRF1 analysis may be due to individual muscular remodelling in mainly slow vs. mixed slow/fast muscles toward the end of prolonged bed rest as reflected by altered myofibre type composition or myofibre type conversion in VL and SOL. Apart from the overall MuRF1 immunosignal expression in the majority of VL and SOL myofibres, overexpression of MuRF1 was detectable only in small subsets of myofibres in both VL and SOL after bed rest. Due to the pre vs. post biopsy paradigm of this study, we cannot comment on the exact time course and possible myofibre type or even muscle-specific expression of MuRF1. The global pattern of MuRF1 detected by immunoblot analysis, however, confirmed that the combined resistive and aerobic countermeasure protocol applied in the WISE study clearly maintained constitutive protein turnover levels in VL and SOL. As compared with subject-matched pre bed rest levels, elevated MuRF1 levels were documented in the VL and SOL from the bed rest-only group after bed rest, suggesting the presence of accelerated proteolysis or high protein turnover rates that would be anticipated from atrophying skeletal muscle due to chronic disuse.

Endothelial NOS3 expression and capillary density in muscle

Aerobic exercise by LBNP prevented maladaptation of the cardiac/vascular system and blood perfusion rates (Dorfman et al. 2007) that should also increase limb blood flow (Joyner & Dietz, 1997) and muscle perfusion and oxygen delivery to the skeletal muscle particularly of the lower extremities following extended bed rest immobilization. We therefore sought to monitor the altered histological pattern of global capillary density in biopsy samples of women in bed rest. The endothelial isoform NOS3, which co-localizes with the endothelial marker PECAM, is a potent vasodilator and mutant mice lacking the gene for this isoform are hypertensive. NO regulates vascular blood flow in humans during voluntary exercise and changes may reduce tolerance for voluntary exercise (Shen et al. 1995). Capillarity reflects the structural potential of the muscle supply (capillary bed) and O₂ and the possible O₂ demand of a muscle, e.g. assessment of aerobic capacity, otherwise known as the capillary-to-fibre interface in muscle (Mathieu-Costello, 1994). We found that the C/F ratio was significantly increased in the trained VL and SOL (BR-EX) as compared with the non-trained BR-CTRL group,

supporting the idea that principally LBNP treadmill exercise, in addition to resistive mechanical load, resulted in higher capillary density patterns and thus muscle perfusion rates in trained vs. non-trained women in bed rest. High-intensity resistance training as performed in the WISE study is known to increase $\text{VO}_{2\text{max}}$ in human skeletal muscle, which is dependent on both limb blood flow and local microperfusion parameters possibly mediated by paracrine actions of sarcolemmal and/or endothelial derived NO (Lau et al. 2000), supporting a separate role for NO in aerobic muscle supply and related force production. Together with previous observations (Rudnick et al. 2004), the present findings provide evidence in women for an effective countermeasure to overcome functional deficits skeletal muscle due to an insufficient capillary-to-fibre interface and thus lowered muscular microperfusion because of the chronic muscle disuse and the haemodynamic fluid shift encountered by the head-down tilt position in bed rest.

Gender differences

We found that muscle atrophy in female bed rest appears to be less distinctive (10–20% loss in SOL) than in males (30–40% loss in SOL), which might be explained by the duration of bed rest, i.e. 60 days (WISE study) vs. 90 days (LTBR study), and the different time points of the pre vs. post biopsy samplings (Rudnick et al. 2004). The role of endocrine mechanisms that could possibly protect skeletal muscle from disuse-induced atrophy in women and men remains to be established. Only a few studies have reported on neuromusculoskeletal system changes in female bed rest immobilization and exercise countermeasure (Ito et al. 1994; Yamamoto et al. 1997; Koryak, 1998). Of the findings reported to date, neural activation and contractile properties of triceps muscle are different in men and women following 120 days of bed rest (Koryak, 1998). Gender differences in strength and muscle fibre characteristics have been previously documented in women and men (Hakkinen et al. 2001). The normal fibre type composition in untrained young men and women (aged 21 yrs) revealed larger CSA values in all major fibre types in men vs. women, myofibre type IIA being the largest in men ($\text{IIA} > \text{I} > \text{IIB}$) and type I being the largest in women ($\text{I} > \text{IIA} > \text{IIB}$; Simoneau et al. 1985). Altered biochemical characteristics were also found between bed rest men and women (Pincivero et al. 2001). The structural muscle changes between men and women following heavy resistive exercise may be subtle (Delmonico et al. 2005). Unlike in men, women (aged 39 ± 3 years) seemed to be hypertrophically responsive, particularly with regard to size of myofibres II, to a maximal resistive 6-month training protocol for maximal strength and power development (Colliander & Tesch, 1991).

Of more than about 500 candidates tested so far in bed rest studies, fewer than 20% are women (Ito et al. 1994; Yamamoto et al. 1997; Koryak, 1998). Therefore, comparable

bed rest data on gender differences in muscle cell biology are still missing and efficacy of reliable countermeasure protocols supporting the neuromusculoskeletal system both in women and in men have to be evaluated following prolonged body deconditioning in bed rest or spaceflight. Future bed rest studies with men and women with and without exercise countermeasure may help to gain further insight into gender-specific cellular adaptation mechanisms.

A potential limitation of our study is the fact that we only studied pre vs. post muscle biopsies instead of a protocol with more biopsies particularly from early onset of muscle atrophy at 1–4 weeks after the start of bed rest. Thus, the exact time course of atrophy including early onset and ongoing cellular remodelling mechanisms and the physiological adaptations in muscle associated with bed rest in women could not be studied. Alternatively, short- and medium-term bed rest studies lasting, for example, 5 and 20 days are planned that might help to answer questions regarding initial responses in human skeletal muscle atrophy due to bed rest-induced disuse. Due to the combined exercise protocol applied during the WISE study we cannot comment on the different modes of resistive vs. aerobic training and their specific benefits for muscle histology, protein turnover/degradation, and precise muscular biomarker responses of women in bed rest.

Conclusion

Molecular biomarkers monitoring the morphological and biochemical changes in defined functional compartments in human skeletal muscle may help provide a better understanding of the cellular responses (e.g. NOS/NO signalling and ubiquitin proteolysis pathways) to prolonged muscle disuse with or without exercise countermeasure in future bed rest studies. Combined protocols using various modes of exercise might be useful as gender-independent potential countermeasures to offset muscle atrophy following body deconditioning in variable clinical settings as well as in human spaceflight.

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